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Dopamine D_3 Receptor Gene: Organization, Transcript Variants, and Polymorphism Associated With Schizophrenia

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DNA fragments from a genomic library were used to establish the partial structure of the human dopamine D_3 receptor gene (DRD3). Its coding sequence contains 6 exons and stretches over 40,000 base pairs. The complete DRD3 transcript and three shorter variants, in which the second and/or third exon are deleted, were detected in similar proportions in brains from four controls and three psychiatric patients. The *Msp* I polymorphism was localized in the fifth intron of the gene, 40,000 base pairs downstream the *Bal* I polymorphism and a PCR-based method was developed for genotyping this polymorphism. The distributions of the *Msp* I and *Bal* I genotypes were not independent in 297 individuals ($\chi^2 = 10.5$, df = 4, $P = 0.03$), but only a weak association was found between allele 1 of the *Bal* I polymorphism and allele 2 of the *Msp* I polymorphism ($\chi^2 = 3.99$, df = 1, $P = 0.04$). The previously reported association between homozygosity at both alleles of the *Bal* I polymorphism and schizophrenia was presently maintained in an extended sample, comprising 119 DSM-III-R chronic schizophrenics and 85 controls ($\chi^2 = 5.3$, df = 1, $P = 0.02$) and found more important in males than in females. The presence of the *Bal* I allele 2 is associated with an early age at onset, particularly in males (df = 35, t value = 2.6, $P = 0.014$). In the same sample, allelic frequencies, genotype counts, and proportion of homozygotes for the *Msp* I polymorphism did not differ between schizophrenics and controls ($\chi^2 = 0.06$, df = 1, $P = 0.80$, $\chi^2 = 0.22$, df = 1, $P = 0.90$ and $\chi^2 = 0.16$, df = 1, $P = 0.69$, respectively). The large distance of the *Msp* I polymorphism from the *Bal* I polymorphism and its localization in the 3′ part of the gene may explain the discrepant results obtained with the two polymorphisms.

KEY WORDS: alternative splicing, splicing junction, *Bal* I polymorphism, *Msp* I polymorphism, polymerase chain reaction

INTRODUCTION

The dopamine D_3 receptor is a target for typical and atypical antipsychotics, localized in brain structures controlling various aspects of motivated behavior, emotions, and reward, and thus potentially of major interest for etiopathology and treatment of schizophrenia [see Schwartz et al., 1992; Sokoloff et al., 1993 for reviews]. In addition, a selective loss of D_3 receptor gene (DRD3) transcript in cortical brain regions [Schmauss et al., 1993] and elevated D_3 receptor binding in the mesolimbic system of schizophrenics were recently reported [Gurevich et al., 1994]. These features make the DRD3 a candidate for genetic studies of schizophrenia, for which evidence from family, twin and adoption studies suggests a significant genetic contribution [McGuffin, 1988].

The DRD3 [Giros et al., 1990] was localized in the q13.3 region of the third chromosome [Le Coniat et al., 1991]. Shorter gene transcript variants, of which the biological function is unknown, have been described in rat [Giros et al., 1991], mouse [Fishburn et al., 1993], and human [Giros et al., 1990; Snyder et al., 1991; Schmauss et al., 1993] brains. In the absence of any structural information on the DRD3, these transcript

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variants were presumed to result from alternative RNA splicing.

A *Bal* I Restriction Fragment Length Polymorphism (RFLP) was identified in the coding region of the DRD3, which corresponds to a point mutation exchanging a Ser to a Gly residue in the N-terminal part of the receptor [Lannfelt et al., 1992]. Two other polymorphisms, leading to RFLPs with *Msp* I and *Pvu* II, were also identified, but not localized in the gene [Sabaté et al., 1994]. Linkage studies of DRD3 polymorphisms with schizophrenia gave consistent negative results [Wiese et al., 1993; Coon et al., 1993; Sabaté et al., 1994], excluding that a mutation at this locus is a major factor in the susceptibility to the disease in the families studied. However, in two independent association studies with French and UK samples [Crocq et al., 1992], an excess of homozygotes for both alleles of the *Bal* I polymorphism at DRD3, reflecting a departure from the Hardy-Weinberg equilibrium, was found in schizophrenic patients, suggesting that the DRD3 may have subtle influence on the liability to develop schizophrenia. This result was replicated, however, with a less marked effect [Mant et al., 1994] and, remarkably, the excess of homozygosity was particularly important in patients whose familial history of the disease was established [Nimgaonkar et al., 1993; Mant et al., 1994]. Nevertheless, these findings could not be reproduced in other studies in Swedish [Jönsson et al., 1993], German [Nöthen et al., 1994], Chinese samples [Yang et al., 1993], or French samples [Sabaté et al., 1994; Laurent et al., 1994].

We have presently studied the structure and the organization of the DRD3, characterized its transcripts in the brain of schizophrenics and controls, localized the *Msp* I polymorphism and developed a PCR-based method to study it. We have also extended our association study with schizophrenia and compared the results obtained with the *Bal* I and *Msp* I polymorphisms.

MATERIAL AND METHODS

Genomic Library Screening and Analysis of Genomic Fragments

A human genomic DNA library (Clontech Laboratories, Inc., Palo Alto, CA) constructed from *Sau* 3A-partially digested DNA in λEMBL3 phage was screened with a [^{32}P]labeled rat D_3 receptor full-length cDNA [Sokoloff et al., 1990]. Hybridization was performed on nitrocellulose filters in 40% formamide, 1 × Denhardt's, 20 mM Tris-HCl, pH 7.4, 4 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 20 μg/ml salmon sperm DNA, and 20 μg/ml Yeast tRNA. Filters were washed at 42°C in 2 × SSC, 0.1% SDS and then in 0.2 × SSC, 0.1% SDS and exposed for 1 night at −80°C with intensifying screens. Eight positive clones HG 1 to HG 44 (see Fig. 1) were isolated and analyzed by restriction endonuclease mapping and Southern blotting using a [^{32}P]labeled cDNA probe encompassing the whole DRD3 cDNA, [^{32}P]labeled *Bam* HI-*Ava* I or *Sac* I restriction fragments of this gene [Giros et al., 1990], or various [^{32}P]labeled oligonucleotides, synthesized from the DRD3 cDNA sequence. Hybridization with radiolabeled oligonucleotides was performed in 1 × Denhardt's, 50 mM Tris-HCl pH 7.4, 6 × SSC, 0.05% NaPPi, and 100 μg/ml Yeast tRNA for 4 h at 42°C. Filters were washed for 20 min in 6 × SSC, 0.05% NaPPi at 37°C, 42°C, and 50°C, successively. DNA fragments of interest were subcloned in pGEM-4Z vector (Promega Corporation, Madison, WI) and sequenced [Sanger et al., 1977]. Restriction mapping of high molecular weight genomic DNA isolated from leukocytes of a Caucasian male was also performed using a [^{32}P]labeled full-length cDNA probe.

Analysis of Gene Transcripts

Total RNA (2 to 5 μg), extracted [Chomczynski and Sacchi, 1987] from various human brain tissues was reverse transcribed using AMV-reverse transcriptase (20 U, Boehringer Mannheim, Mannheim, Germany), primer 1 (5′-GGTCTAGATCCGTCTCCTTTGTTTC-AGCA-3′) and amplified for 35 cycles (92°C, 56°C, 72°C, 1 min each) using Taq polymerase (2.5 U, Perkin-Elmer, Roche, Inc., Branchburg, NJ) with primers 1 and 2 (5′-CGAATTCATGGCATCTCTGAGCCAGCTGAGTA-3′) in 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 M dithiotreitol, 0.01% gelatin, and 100 μM of each dNTP or reverse transcribed using primer 3 (5′-ACG-GAATTCAGATCTTCTGCTCCCTCAGCAAGACAG-3′) and amplified using primers 3 and 4 (5′-AAAGGAGACGGAAAAGGAT-3′). RT-PCR products were electrophoresed through 2% agarose, 1% Nuseive (FMC Bioproducts, Rockland, ME) gel, transferred to nitrocellulose filters, and hybridized with [^{32}P]labeled oligonucleotides (A: 5′-ATGATGTGTACAGCCAGC-3′, B: 5′-GGAGTGACTGTCCTT GTCTATGCCAGA-3′ or C: 5′-TCCCTGAGTCCCACCATA-3′) (10^6 cpm/ml) as described above. RT-PCR products obtained with primers 2 and 5 (5′-TTTGTTGGGGGAAGCCAG-3′) were amplified and subcloned into M13mp19 (Promega Corporation, Madison, WI) for sequencing [Sanger et al., 1977].

For quantifying the expression of the various gene transcripts, PCR products obtained as above with primers 1 and 2 in the presence of [^{32}P]dCTP were electrophoresed through 6% polyacrylamide gel or through 2% agarose, 1% Nusieve gels, which were dried and then exposed to X-ray films. Autoradiograms were scanned with an absorbance detector (ISCO UA-5, Roucaire, Paris, France) coupled to a computing integrator (SP 42000, Spectra-Physics). Radioactivity level of the DNA fragments were corrected for their respective size.

The position of the various primers and oligonucleotide probes on the DRD3 cDNA sequence is indicated in Figure 1.

DNA Genotyping of the *Bal* I and *Msp* I Polymorphisms

Venous blood samples were collected in ethylenediaminetetracetic acid-containing tubes and DNA was extracted from peripheral leukocytes [Kunkel et al., 1977]. The *Bal* I polymorphism was genotyped by PCR as previously described [Lannfelt et al., 1992]. For genotyping the *Msp* I polymorphism, genomic DNA was amplified by PCR, carried out with the primers 6: 5′-CTGTCTGGTACATATTGGATA-3′ and 7: 5′-GAGATG-GAGTCTTGCTCTGTC-3′, in a total volume of 25 μl containing 10–50 ng of genomic DNA in 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 5 pmol of each

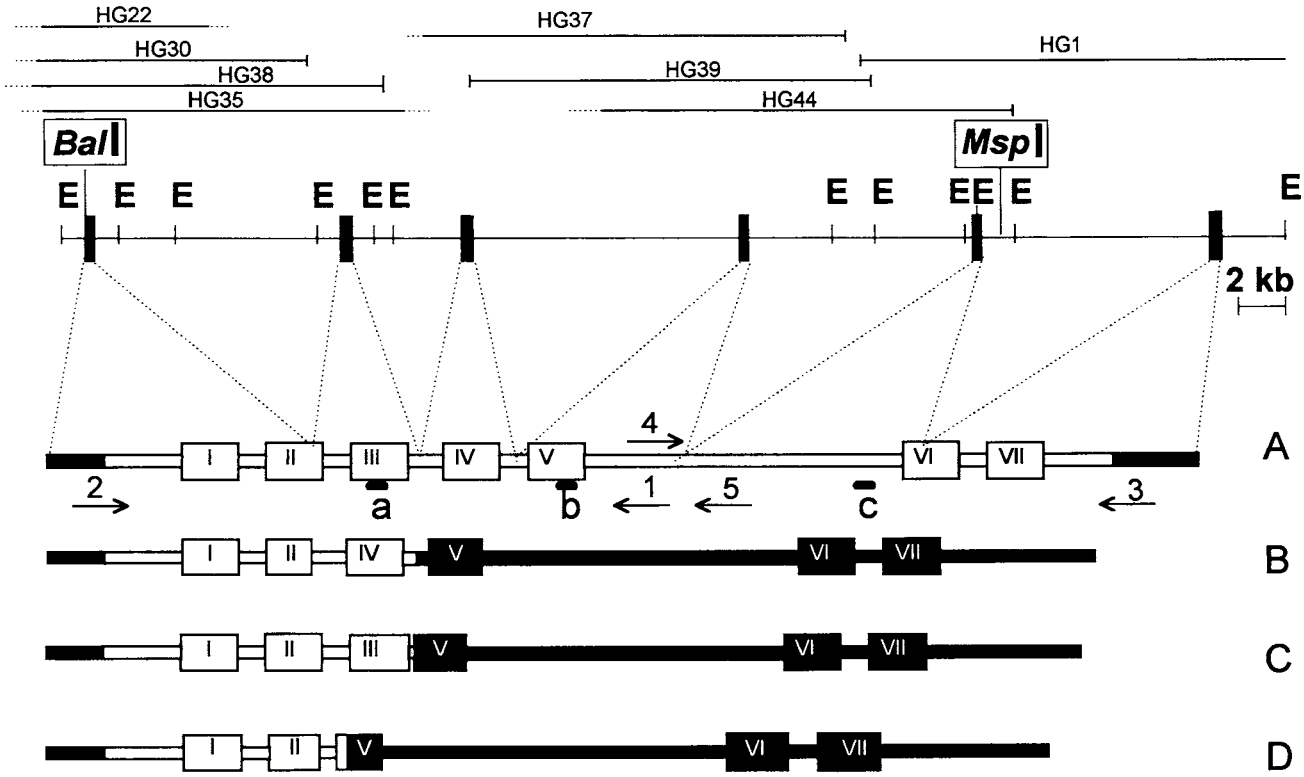

Fig. 1. Diagrams of the DRD3 gene and spliced transcript variants. The upper portion represents the restriction map with *Eco* RI endonuclease (E), the solid boxes indicate the exons, and the upper lines the genomic fragments present in λDNA clones used to establish the map and the intron/exon junctions. Interrupted lines indicate uncertainty at ends. *Bal* I and *Msp* I indicate the position of the polymorphic restriction sites. The lower part shows the structure of the spliced gene transcript variants. Boxes represent transmembrane domains I–VII. Open lines indicate coding sequences, while dark lines are noncoding sequences. A is the full-length transcript and B, C, and D are shorter variants. In C and D, the deletions introduce a shift in the reading frame, leading to very short coding sequences. The position of the primers used in PCR experiments is indicated by the arrows in the full-length cDNA (A), while thick dark lines locate the position of the oligonucleotides used as probes. Genomic sequence and structure reported in this study are available from GenBank under the accession number U25441.

primer, 100 μM of each dNTP (dATP, dCTP, dGTP, and dTTP), 0.5 mM dithiothreitol, Taq polymerase (1.5 U), and 0.01% gelatin for 35 cycles (92°C for 1 min, 56°C for 1 min, and 72°C for 1 min). The amplified DNA was digested with *Msp* I (5 U, Boehringer Mannheim, Mannheim, Germany), in a total volume of 15 μl, electrophoresed through 2% agarose, and visualized using ethidium bromide staining. In preliminary experiments, we determined that the amount of *Msp* I used was in a ten-fold excess.

Subjects

For analysis of DRD3 transcripts, post-mortem brain tissues were obtained from Hôpital de la Salpétrière, Paris, France (normal brain 882). Other brains (three controls and three from psychiatric patients) were collected by the Maryland Psychiatric Research Center, Baltimore, MD by consecutive cases at the Coroner's Office upon consent of family. Clinical status was established with consensus of at least two psychiatrists according to DSM-III R and post-mortem delays were from 3 to 22 h.

We extended the previously reported association study [Crocq et al., 1992] by enlarging the sample. Inpatients and controls in the whole sample were recruited from a single hospital in Alsace. Diagnoses were established according to DSM-III-R criteria. A consensus diagnosis was established by two psychiatrists (MAC, FD), prior to genetic analysis. Subjects were not included into the study when the two psychiatrists disagreed. The schizophrenic sample was recruited opportunistically among the inpatients who were sejourning in the hospital at the time of the study and who met DSM-III-R criteria for chronic schizophrenia. Other diagnostic categories were recruited by consecutive admissions. No attempt was made to match the patients to controls for age and sex distributions. All persons were of French or Alsatian ancestry; Mediterranean Caucasians, Asians and Africans were excluded. A total sample including 85 controls, 119 schizophrenics, 28 unipolar depressives, 39 patients with bipolar disorder, 16 with DSM-III-R personality disorder, ten healthy first-degree relatives of psychiatric patients was used for comparing the distributions of the *Msp* I and *Bal* I

genotypes. All schizophrenic patients had shown for at least 2 years continuous signs of illness, including cognitive and functional deficits. Family history among first-degree relatives could be assessed from 61 patients and age of onset of the disease from 88 patients. Analysis of gene transcripts and genotyping was performed blind to the clinical status of individuals. The study was performed after agreement of a local Ethical Committee and informed patients' consent.

RESULTS

D_3 Receptor Gene Structure

The structure of the DRD3 was deduced from analysis of eight λDNA clones (HG1-44) isolated from a human genomic library and human genomic DNA (Fig. 1A), initially screened using a rat D_3 receptor full-length cDNA as a probe [Sokoloff et al., 1990]. Restriction mapping of the positive phages was subsequently performed with a human D_3 receptor cDNA probe [Giros et al., 1990] or oligonucleotide probes synthesized from this sequence. Using the structure of the rat DRD3 as a guideline, we identified and sequenced restriction fragments containing putative intron/exon splicing junctions. To establish the structure of the DRD3, ambiguous positions or ordering of the restriction fragments of isolated phages were clarified by restriction mapping of human genomic DNA.

The coding sequence of the DRD3 consists in five exons, dispersed over more than 53,000 base pairs (bp). The exons grossly correspond to functional domains of the D_3 receptor. Exon 1 spans the putative transmembrane domains (TMs) I and II, exon 2 the TM III, exon 3 the TM IV, exon 4 the TM V, exon 5 the third intracytoplasmic loop and exon 6, the TMs VI and VII, and 3′ untranslated region and the approximate size of the corresponding introns are 10,000, 4,500, 12,000, 11,000 and 10,000 bp, respectively. Each intron contains an acceptor/donor consensus sequence of the GT/AG form (Table I). A consensus sequence for a putative splice acceptor was found 34 bp before the initial translation ATG codon (Table I).

Gene Transcripts Variants

Using reverse transcription-PCR (RT-PCR), we have sought for synthesis of DRD3 transcript variants, as it is the case for rodent D_3 receptor gene products [Giros et al., 1991; Fishburn et al., 1993]. RT-PCR was performed using primers 5 and 2, located at the 5′ end of the D_3R coding sequence and at the beginning of the third intracytoplasmic loop (Fig. 1), and RNA from various human brain tissues. Four bands of 661 bp (A), 548 (B), 518 (C), and 405 (D) bp were generated (Fig. 2). After reamplification with primers 1 and 2 of cDNA obtained from a control brain (882), the four different PCR products were subcloned and sequenced. We found that the 661 bp band corresponds to the complete coding sequence. The 548 bp band results from the amplification of a transcript with a 113 bp deletion, corresponding to the exclusion of the second exon. The 518 bp band results from the amplification of a transcript deleted of 143 bp, corresponding to the exclusion of the third exon. Finally, the 405 bp band corresponds to a transcript without second and third exons (deletion of 256 bp). All these deletions introduce a shift in the reading frame, indicating that the three shorter transcripts potentially code for very small proteins, constituted by the N-terminal part of the protein, TMs I, II, and IV (transcript B), the TMs I, II, and III (transcript C), or the TMs I, II, and V (transcript D) of the D_3 receptor.

Amplification with primers 3 and 4, located at the beginning of the third intracytoplasmic loop and at the 3′ end of the coding sequence respectively (Fig. 1), results in a unique band of 566 bp, corresponding to the complete transcript. No additional transcript was observed after reamplification. No larger transcript, resulting from insertion, was found after amplification with primers 1 and 2, nor with primers 3 and 4.

PCR products from DRD3 transcript variants were quantified in various brain regions from normal and psychiatric patients. As shown in Table II, the complete transcript, as well as the three variants could be detected in both normal (n = 4) and psychiatric (n = 3) brains. In some cases, amplification was found difficult to achieve, so the transcripts could not be quantified in all brain regions from all individuals. Regional differences were found between regions for one normal brain (60), but not another normal brain (882). In most cases, the complete transcript is the major one, the only exception being found in the nucleus accumbens from one normal brain (60) and one schizophrenic brain (262). Owing to the low size of the samples, to their ethnical diversity, and to variations between brain regions, no attempt has been made to compare statistically control and psychiatric patients.

PCR-Based Analysis of the *Msp* I Polymorphism

In order to develop a PCR-based method for genotyping the *Msp* I polymorphism, that we originally identified in the DRD3 by conventional Southern blotting [Sabaté et al., 1994], we further analyzed some phages isolated from the human genomic library. After digestion by *Msp* I, two clones gave different patterns, when probed with a [^{32}P]labeled *Bam* HI-*Ava* I restriction fragment: Hg 44 digestion led to labeled 3.2 kb and 0.8 kb *Msp* I fragments, whereas HG 1 digestion gave rise

TABLE I. Exon/Intron Junctions in the Human D_3 Dopamine Receptor Gene

5′-end[a]	.....tattgttttctgtctcctcacagGAAGCCCCTTGGCATCACGCACCTCCTCTGGGCT **ATG** GCA TCT CTG AGC
Intron 1	GTG GTA TAC CTG GAGgtgagtagacttcaggtgcatgttgac ..... ctaacatccttgtctctgatacccagGTG ACA GGT GGA GTC
Intron 2	GCC ATC AGC ATA GAC AGgtagggctgcaattcccctt ..... agcctatccctctcccctttgctttttcaagG TAC ACT GCA GTG
Intron 3	GGC TTT AAT ACC ACA G gtaacagtgatggcttcatttc ..... tttaccttcccccttctcatccacagGG GAC CCC ACT GTC
Intron 4	GGC TTC CCC CCA gtaagtaccctggaggggggtag ..... attccctcttgtcacacctgcagACC CTC TCT CCT GAC
Intron 5	GTG GCC ATT GTG CTT Ggtaagtttgggttggcttgagctgtgtt .... cccagtttctctcttatttggcaacttagGG GCC TTC ATT GTC

[a] Sequence upstream of the initial translation ATG codon.

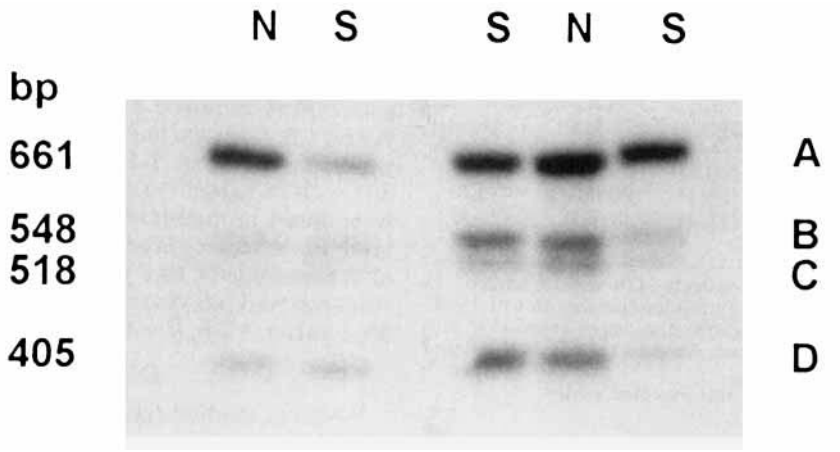

Fig. 2. Analysis of DRD3 transcript variants by reverse transcription-PCR in various human brain regions. Total RNA from caudate nucleus (lanes 1 and 2), accumbens (lanes 3 and 4), or mammillary bodies (lane 5) from brain of normal individual (N) or schizophrenic patient (S) were reverse-transcribed and amplified by PCR using primers 1 and 3. PCR products were electrophoresed, blotted and hybridized with [^{32}P]labeled oligonucleotide A.

to a unique labeled fragment of 1.6 kb. Therefore, HG 1 and HG 44 could contain respective allelic DNAs. A 1.4 kb *Eco* RI restriction fragment of HG 1 and a 0.8 kb *Msp* I restriction fragment of HG 44 were subcloned and sequenced. The sequences were identical in these fragments with a few exceptions. A substitution for a G was found in phage HG 1, at the level of the 3′-*Msp* I site of the phage HG 44. Six to forty bp upstream this *Msp* I site, we found additional sequence variations between the HG 1 and HG 44 phages, consisting in substitutions and deletions. This presumably polymorphic *Msp* I site is located 532 bp after the beginning of the fifth intron, 40,000 bp downstream the previously identified *Bal* I polymorphism (Fig. 1).

Primers were designed from flanking sequences of the putative polymorphic *Msp* I site and a 396 bp segment was amplified by PCR from DNAs of unrelated individuals and digested by *Msp* I. The *Msp* I site was found polymorphic: depending on the absence or the presence of this site, either a fragment of 396 bp or two fragments of 252 and 144 bp were produced. On a large sample of controls and psychiatric patients (N = 297), the frequencies were 52% for allele 1 (absence of the *Msp* I site) and 48% for the allele 2 (presence of the *Msp* I site), which led to a Polymorphic Information Content (PIC) value of 0.37.

The distributions of the *Msp* I and the *Bal* I genotypes were not independent in this sample ($\chi^2 = 10.5$, df = 4, $P = 0.03$). The associations of haplotypes [*Bal* I (1-2)-*Msp* I (1-1)] and [*Bal* I (1-1)-*Msp* I (2-2)] were observed more often than expected from random mating (Table III). The haplotype [*Bal* I (1-1)-*Msp* I (1-1)] is observed less often than expected (Table III). Haplotype frequencies were estimated according to the general method of Mattiuz and colleagues [1970]. There exists a weak association between the *Bal* I allele 1 and the *Msp* I allele 2, and, similarly, between the *Bal* I allele 2 and the *Msp* I allele ($\chi^2 = 3.99$, df = 1, $P = 0.04$).

Association of D_3 Receptor Gene Polymorphisms With Schizophrenia

We extended to a larger sample a previously published study [Crocq et al., 1992]. Forty-six chronic schizophrenics and fourteen controls were added to the

TABLE II. Analysis of the Four DRD3 Transcripts in Various Regions of Human Brain From Normal and Schizophrenic Individuals

Brain region	Subject number	Clinical status[a]	Age/race/sex[b]	Relative proportion of transcript variants			
				A	B	C	D
Caudate nucleus	882	N	65/Fr/F	73	12	9	6
	48	N	45/B/M	75	9	3	13
	60	S	28/W/F	43	16	10	31
Putamen	882	N		80	10	6	4
Nucleus accumbens	73	N	24/W/M	45	22	13	20
	60	S	28/W/F	6	10	10	74
	68	SA	35/W/F	40	25	7	28
	262	S	42/B/M	2	14	8	76
Olfactory tubercle	882	N		74	12	8	6
Mammillary bodies	882	N		68	16	10	6
	81	BI	31/B/M	72	15	5	8

[a] N, normal; S, schizophrenic; SA, schizoaffective; BI, bipolar.
[b] Fr, French; W, White American; B, Black American. Transcript A corresponds to the full coding sequence whereas transcripts B, C, and D correspond to shorter variants in which exons 1, 2, and 1 + 2, respectively, are excluded.

TABLE III. Distributions of *Bal* I and *Msp* I Genotypes in the Total Sample*

Bal I genotypes	*Msp* I genotypes			
	1-1	1-2	2-2	Totals
1-1	26[a] (35.2)	69 (68.6)	39[a] (30.2)	134
1-2	43[a] (34.9)	65 (68.1)	25 (30.0)	133
2-2	9 (7.9)	18 (15.4)	3 (6.8)	30
Totals	78	152	67	297

* *The total sample* included 85 controls, 119 schizophrenics, 39 unipolar depressives, 28 patients with bipolar disorder, 16 with DSM-III-R personality disorder, and 10 healthy first-degree relatives of psychiatric patients. Numbers in brackets are expected values calculated assuming a random mating.
[a] These values significantly depart from expected values.

previous sample. The distributions of the *Bal* I 1-1, 1-2, and 2-2 genotypes were (20, 19, 7) and (7, 5, 2) in the additional schizophrenics and controls, respectively. The low size of the additional sample does not allow a meaningful separate analysis. In the combined sample (Table IV), there was a significant, albeit smaller than reported in the first study, excess of homozygotes for both *Bal* I alleles among schizophrenics as compared to controls by the Woolf's method [Woolf, 1955] ($\chi^2 = 5.3$, df = 1, $P < 0.02$). Homozygosity was associated with a slightly increased risk of schizophrenia: the odds ratio was 1.94, with a 95% confidence interval of 1.1–3.4, as calculated as described in Sachs et al. [1992]. No significant differences in the frequencies of *Bal* I alleles were noted in schizophrenics as compared to controls.

The allele frequencies and the distributions of the genotypes for the *Msp* I polymorphism were compared in the same samples of controls and schizophrenic patients (Table IV). There was no differences in the *Msp* I allele frequencies ($\chi^2 = 0.97$, df = 1, $P = 0.30$) between controls and schizophrenics and no differences were found in the distributions of *Msp* I genotypes.

A slightly larger sample (125 schizophrenics and 101 controls), comprising some individuals genotyped for the *Bal* I polymorphism and not for the *Msp* I polymorphism, was analysed for influence of sex, family history and age of onset. In this sample, the excess of *Bal* I homozygotes among schizophrenics was also significant ($\chi^2 = 3.8$, df = 1, $P = 0.05$). The excess of homozygotes was more important in 36 male schizophrenics as compared to 50 male controls ($\chi^2 = 3.0$, df = 1, $P = 0.08$) than in 89 female schizophrenics as compared to 51 female controls ($\chi^2 = 0.58$, df = 1, $P = 0.44$). The proportion of homozygotes did not differ among schizophrenics with (N = 36) or without (N = 89) a family history of schizophrenia among first-degree relatives ($\chi^2 = 0.36$, df = 1, $P = 0.55$). In addition, the *Bal* I allele 2 is associated with a younger age at onset; 2-2 homozygotes, first required treatment at mean age of 20.2 years, 1.6 years earlier than 1-2 heterozygotes, and 3.5 years earlier than 1-1 homozygotes (unpaired t-test, d.f. = 52, t-value = 1.8, $P = 0.077$). These differences were more pronounced when only males were considered: male schizophrenics with the 2-2 genotype fell ill at a mean age of 18.1 years, 3.8 years earlier than 1-2 subjects, and 5.6 years earlier than 1-1 subjects (d.f. = 35, t-value = 2.6, $P = 0.014$).

DISCUSSION

We have studied the structure of the DRD3 and provide information on how gene transcript variants, either previously detected [Giros et al., 1991; Snyder et al., 1991; Schmauss et al., 1993] or presently characterized in brain from normal individuals and psychiatric patients may be produced. In addition, we report that the association of homozygosity at the *Bal* I polymorphism and schizophrenia, already observed [Crocq et al., 1992], is maintained in a larger sample, whereas no association is found with the *Msp* I polymorphism, a feature possibly related to the distance existing between these two genetic markers.

The human DRD3, like the homologous D_2 [Grandy et al., 1989] and D_4 [Van Tol et al., 1991] receptor genes, as well as its rat [Giros et al., 1991] and mouse [Fishburn et al., 1993] counterparts, has its coding sequence interrupted by introns, that makes the whole gene stretched over more than 53,000 bp.

It should be noted that a consensus sequence for splice acceptor is found 34 bp upstream the initiation ATG codon, raising the possibility of one or more additional exons to be identified at the 5′-end of the DRD3. An intron at the same position was found in the rat D_2 receptor gene [Minowa et al., 1992; Valdenaire et al., 1994], and similar consensus sequences were found in the human D_2 [Grandy et al., 1989], as well as rat D_3 receptor genes [Giros et al., 1991].

This gene organization could lead to the production of different transcripts generated by alternative splicing. A transcript variant has been identified in the case of the D_2 receptor gene, differing by the exclusion of the sixth exon [Giros et al., 1989; Dal Toso et al., 1989; Monsma et al., 1989; Selbie et al., 1989]. In the case of the DRD3, several shorter variant transcripts were identified in the rat [Giros et al., 1991; Snyder et al., 1991], mouse [Fishburn et al., 1993], and human [Giros

TABLE IV. *Bal* I and *Msp* I Genotypes in Controls and Schizophrenics

Polymorphism and sample	N	Frequency of allele 1	Genotypes counts and frequency		
			1-1	1-2	2-2
Msp I Polymorphism					
Controls	85	0,53	23 (0.27)	44 (0.52)	18 (0.21)
Schizophrenics	119	0,52	29 (0.24)	65 (0.55)	25 (0.21)
Bal I Polymorphism					
Controls	85	0,66	33 (0.39)	46 (0.54)	6 (0.07)
Schizophrenics	119	0,67	57 (0.48)	45 (0.38)	17 (0.14)

et al., 1990; Schmauss et al., 1993] brains, which not always correspond to the exclusion of a complete exon; some seem produced by RNA splicing at a level of exonic sequences. We presently identified three shorter variant transcripts corresponding to deletions of complete exons, i.e., exons 2, 3, or both. In contrast, we could not detect the transcript variant identified by Schmauss and colleagues [1993] that corresponds to the exclusion of a partial exon since no intron was found in the gene at this level. This discrepancy may be related to the use of a different set of primers, or to different PCR experimental conditions, suggesting peculiar features and/or very low abundance of this transcript variant. The presently identified deletions introduce shifts in the open reading frame, so that the proteins potentially produced from these shorter transcripts are very small and probably lack any direct biological activity. As already suggested [Giros et al., 1991], they could nevertheless be involved in the control of active D_3 receptor abundance, a mechanism that might be disturbed in psychiatric disorders.

This possibility was explored by studying the production of the gene transcript in some post-mortem brains from psychiatric patients. The complete transcript, as well as the three shorter variants, could be detected in several areas of all psychiatric brains examined, suggesting that the splicing mechanisms at DRD3 are not qualitatively disturbed. This does not rule out a quantitative disturbance. The expression of these transcripts seemed variable among regions and individuals, but no clearcut differences could be evidenced between normal and disease brains, however with a limited number of brains examined. The variable amplification of the gene transcripts, presumably related to variable tissue preservation, and the possible variable yields of PCR products, make difficult the actual comparison between brains. Hence, it might be important to examine further this question using larger samples and various schizophrenic subgroups.

We presently observed that the association of homozygosity at either allele of the *Bal* I polymorphism with schizophrenia is maintained in an extended sample. This association was modest, as observed in other studies [Crocq et al., 1992; Mant et al., 1994; Mingaonkar et al., 1993]. In order to improve the homogeneity of the schizophrenic sample, we included only inpatients who met DSM-III-R criteria for chronic course (i.e., at least 2 years of illness). In this respect, our sample may differ from other published studies, which also included outpatients, who, as a group, have less cognitive and functional deficits than chronic patients. In contrast with other studies [Nimgaonkar et al., 1993; Mant et al., 1994], no differences were found between patients with or without a family history. In agreement with a previous study [Mant et al., 1994], our data indicate that the excess of *Bal* I homozygosity is more pronounced in male schizophrenics as compared to females. In addition, 2-2 homozygosity is associated with an early age of onset, particularly among male schizophrenics. As stated in DSM-IV (American Psychiatric Association, Washington DC, 1994), age at onset may have a pathophysiological significance. Individuals with an early age at onset are more often male, have more evidence of structural brain abnormalities, more prominent negative signs and symptoms, more evidence of cognitive impairment, and a worse outcome. Hence, our data suggest that the presence of *Bal* I allele 2 might be associated with an increased vulnerability to a form of schizophrenia characterized by early onset, chronicity of illness, and poor cognitive performances.

No significant association was found between the *Msp* I polymorphism and schizophrenia, as observed in our earlier study with a distinct sample [Sabaté et al., 1994]. The distributions of *Bal* I and *Msp* I genotypes were not independent, as expected for two markers in the same locus, but the association of *Bal* I allele 1 and *Msp* I allele 2 was very weak, most likely due to physical distance (35,000 bp) between them. Since the association of homozygosity at the *Bal* I polymorphism and schizophrenia is also modest, it is not surprising that no significant association was found between the *Msp* I polymorphism and schizophrenia. This suggests that the *Bal* I polymorphism may be closer to a deleterious mutation than the *Msp* I polymorphism. The recent observation [Crocq et al., unpublished results] that the frequency of the *Bal* I allele 1 is as low as 0.12 in 54 unrelated Congolese individuals, as compared to 0.6–0.7 in Caucasian and Asiatic populations, indicates that the mutation at the *Bal* I site is not deleterious by itself. It is therefore tempting to seek for other polymorphisms in the 5′-region of the DRD3, in order to select other markers more accurate for genetic studies of schizophrenia.

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REFERENCES

Chomczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidinium thicyanate-phenol-chloroform extraction. Analyt Biochem 162:156–159.

Coon H, Byerley W, Holik J, Hoff M, Myles-Worsley M, Lannfelt L, Sokoloff P, Schwartz JC, Waldo M, Freedman R, Plaetke R (1993): Linkage analysis of schizophrenia with five dopamine receptor genes in nine pedigrees. Am J Hum Genet 52:327–334.

Crocq MA, Mant R, Asherson P, Williams J, Hode Y, Mayerova A, Collier D, Lannfelt L, Sokoloff P, Schwartz JC, Gill M, Macher JP, McGuffin P, Owen MJ (1992): Association between schizophrenia and homozygosity at the dopamine D_3 receptor gene. J Med Genet 29:858–860.

Dal Toso R, Sommer B, Ewert M, Herb A, Prichett DB, Bach A, Shivers BD, Seeburg PH (1989): The dopamine D_2 receptor: two molecular forms generated by alternative splicing. EMBO J 8:4025–4034.

Fishburn CS, Bellili D, David C, Carmon S, Fuchs S (1993): A novel short isoform of the D_3 dopamine receptor generated by alternative splicing in the third cytoplasmic loop. J Biol Chem 268:5872–5878.

Giros B, Sokoloff P, Martres MP, Riou JF, Emorine LJ, Schwartz JC (1989): Alternative splicing directs the expression of two D_2 dopamine receptor isoforms. Nature 342:923–926.

Giros B, Martres MP, Sokoloff P, Schwartz JC (1990): cDNA cloning of the human dopaminergic D_3 receptor and chromosome identification. CR Acad Sci (Paris) 311:501–508.

Giros B, Martres MP, Pilon C, Sokoloff P, Schwartz JC (1991): Shorter variants of the D_3 dopamine receptor produced through various patterns of alternative splicing. Bioch Biophys Res Commun 176:1584–1592.

Grandy DK, Marchionni MA, Makan H, Stofko RE, Alfano M, Frothingham L, Fischer JB, Burke-Howie KJ, Bunzow JR, Server AC, Civelli O (1989): Cloning of the cDNA and gene for a human D_2 dopamine receptor. Proc Natl Acad Sci USA 84:9762–9766.

Gurevich EV, Kung MP, Bordelon Y, Joyce JN (1994): Dopamine D_3 receptors are elevated in mesolimbic dopamine system of schizophrenics: receptor autoradiographic studies. Neuropsychopharmacology 10:236S.

Jönsson E, Lannfelt L, Sokoloff P, Schwartz JC, Sedvall G (1993): Lack of association in a Bal I polymorphism in the dopamine D_3 receptor gene in schizophrenia. Acta Psychiatrica Scandinavica 87:345–347.

Kunkel LM, Smith KD, Boyer SH (1977): Analysis of human Y chromosme specific reiterated DNA in chromosome variants. Proc Natl Acad Sci USA 74:1245–1249.

Lannfelt L, Sokoloff P, Martres MP, Pilon C, Giros B, Jönsson E, Sedvall G, Schwartz JC (1992): Amino acid substitution in the dopamine D_3 receptor as a useful polymorphism for investigating psychiatric disorders. Psychiatric Genetics 2:249–256.

Laurent C, Savoye C, Samolyk D, Meloni R, Mallet J, Campion D, Martinez M, D'Amato T, Bastard C, Dollfus S (1994): Homozygosity at the D_3 receptor locus is not associated with schizophrenia. J Med Genet 31:260–264.

Le Coniat M, Sokoloff P, Hillion J, Martres MP, Giros B, Pilon C, Schwartz JC, Berger R (1991): Chromosomal localization of the human D_3 dopamine receptor gene. Hum Genet 87:618–620.

Mant R, Williams J, Asherson P, Parfitt E, McGuffin P, Owen MJ (1994): The relationship between homozygosity at the dopamine D_3 receptor gene and schizophrenia. Am J Med Genet 54:21–26.

Mattiuz PL, Ihde D, Piazza A, Cepellini R, Bodmer WF (1970): New approaches to the population genetic and segregation analysis of the HL-A system. In Teraski PI (ed): "Histocompatibility Testing." Copenhagen: Munskgaard, pp 193–205.

McGuffin P (1988): Genetics of schizophrenia. In Bebbington P, McGuffin P (eds): "Schizophrenia, The Major Issues." London: Heinemann, pp 107–126.

Minowa T, Minowa MT, Mouradian MM (1992): Analysis of the promoter region of the rat D_2 dopamine receptor gene. Biochemistry 31:8389–8396.

Monsma FJ, McVittie LD, Gerfen CR, Mahan LC, Sibley DR (1989): Multiple D_2 dopamine receptors produced by alternative RNA splicing. Nature 342:926–929.

Nimgaonkar VL, Zhang XR, Caldwell JG, Ganguli R, Chakravarti A (1993): Association study of schizophrenia with dopamine D_3 receptor gene polymorphisms: Probable effects of family history of schizophrenia? Am J Med Genet (Neuropsychiatr Genet) 48:214–217.

Nöthen M, Körner J, Lannfelt L, Sokoloff P, Schwartz JC, Lanczik M, Rietchel M, Cichon S, Kramer R, Fimmers R, Möller HJ, Beckman H, Propping P (1993): Lack of association between schizophrenia and alleles of the dopamine D_1, D_2, D_3 and D_4 receptor loci. Arch Gen Psych Psychiatric Genetics 3:89–94.

Sabaté O, Campion D, d'Amato T, Martres MP, Sokoloff P, Giros B, Leboyer M, Jay M, Guedj F, Thibaut F, Dollfus S, Preterre P, Petit M, Babron MC, Waksman G, Mallet J, Schwartz JC (1994): Failure to find evidence for linkage or association between the dopamine D_3 receptor gene and schizophrenia. Am J Psychiat 151:107–111.

Sachs L (1992): "Angewandte Statistik, 7. Auflage." Berlin: Springer, pp 310.

Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain-termination inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Schmauss C, Haroutunian V, Davis KL, Davidson M (1993): Selective loss of dopamine D_3-type receptor mRNA expression in parietal and motor cortices of patients with chronic schizophrenia. Proc Natl Acad Sci USA 90:8942–8946.

Schwartz JC, Giros B, Martres MP, Sokoloff P (1992): The dopamine receptor family: Molecular biology and pharmacology. Sem Neurosci 4:99–108.

Selbie LA, Hayes G, Shine J (1989): The major dopamine D_2 receptor: Molecular analysis of the human D_{2A} subtype. DNA 8:683–689.

Snyder LA, Roberts JL, Sealfon SC (1991): Alternative transcripts of the rat and human dopamine D_3 receptor. Biochem Biophys Res Commun 180:1031–1035.

Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC (1990): Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. Nature 347:146–151.

Sokoloff P, Martres MP, Giros B, Lévesque D, Diaz J, Pilon C, Griffon N, Schwartz JC (1993): The dopamine-D_3 receptor. In Niznik HB (ed): "Dopamine Receptors and Transporters." New York: Marcel Dekker, Inc., pp 165–188.

Valdenaire O, Vernier P, Maus M, Dumas Milne Edwards JB, Mallet J (1994): Transcription of the rat dopamine D_2 receptor gene from two promoters. Eur J Biochem 220:577–584.

Van Tol HHM, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, Civelli O (1991): Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. Nature 350:610–614.

Wiese C, Lannfelt L, Kristbjarnarson H, Yang L, Zoega T, Sokoloff P, Ivorsson O, Vinogradov S, Schwartz JC, Moises HW, Helgason T (1993): No linkage between schizophrenia and D_3 dopamine receptor gene locus in Icelandic pedigrees. Psychiatry Res 46: 253–259.

Woolf B (1955): On estimating the relation between blood group and disease. Ann Hum Genet 19:251–253.

Yang L, Li T, Wiese C, Lannfelt L, Sokoloff P, Xu CT, Zeng Z, Schwartz JC, Liu X, Moises HW (1993): No association between schizophrenia and homozygosity at the D_3 dopamine receptor gene. Am J Med Genet (Neuropsychiatr Genet) 48:83–86.